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Review

SNAREopathies: Diversity in Mechanisms and Symptoms

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Neuronal SNAREs and their key regulators together drive synaptic vesicle exocytosis and synaptic transmission as a single integrated membrane fusion machine. Human pathogenic mutations have now been reported for all eight core components, but patients are diagnosed with very different neurodevelopmental syndromes. We propose to unify these syndromes, based on etiology and mechanism, as "SNAREopathies." Here, we review the strikingly diverse clinical phenomenology and disease severity and the also remarkably diverse genetic mechanisms. We argue that disease severity generally scales with functional redundancy and, conversely, that the large effect of mutations in some SNARE genes is the price paid for extensive integration and exceptional specialization. Finally, we discuss how subtle differences in components being rate limiting in different types of neurons helps to explain the main symptoms.

Twenty-five years after their discovery (Schiavo et al., 1995; Söllner et al., 1993), the evidence of a crucial role of SNARE (soluble NSF attachment protein receptor) complexes in many membrane fusion processes in the brain is overwhelming (Südhof and Rothman, 2009). The neuronal SNAREs syntaxin 1, SNAP25, and synaptobrevin/VAMP2 are at the core of a highly conserved, integrated molecular machine that drives synaptic transmission by synaptic vesicle exocytosis (Südhof and Rothman, 2009) and also secretion of neuropeptides and neurotrophins from dense core vesicles (Arora et al., 2017; Shimojo et al., 2015). Strikingly, there is no backup plan for these fusion reactions. Inactivation of single SNARE genes or the SNARE organizer MUNC18-1 abolishes synaptic transmission and even prevents spontaneous fusion events in most neurons studied so far (Schoch et al., 2001; Verhage et al., 2000; Washbourne et al., 2002). Given this lack of redundancy, mutations in the human genes encoding these proteins are expected to result in clinically important effects on brain function. In 2008, the first human patients were described, initially with mutations in *STXBP1* (Saitsu et al., 2008), the human gene encoding MUNC18-1. To date, patients have been described carrying mutations in each neuronal SNARE gene and in five genes encoding important organizers of neuronal SNARE function that together constitute the integrated molecular fusion machine (Figure 1; Table 1). Most reported mutations are heterozygous *de novo* missense or loss-of-function (LoF) mutations (mutations that result in reduced or abolished protein function: [partial] gene deletions, small insertions, truncations, and frameshift or splice-site mutations). The causality of these mutations is now fairly well established, at least in a few of these genes. It could be expected that mutations in any component of this integrated machine lead to similar symptoms. However, recent studies now suggest that mutations give rise to a surprisingly diverse palette of symptoms, with neurodevelopmental delay (NDD) in the domains of speech, language, motor function, and intellectual ability, as the most common aspect. In addition, other neurological features associated with severe developmental delay are observed, such as seizures and epileptiform abnormalities, and neurological motor problems, such as spasms and ataxia (Figure 2; Table 1). Children carrying mutations in a single SNAREopathy gene can have more than 10 different diagnoses, depending on how they entered the health care system (https://stxbp1.cncr.nl/stxbp1_disorders), a situation referred to as "the diagnostic odyssey" for the families and caretakers. Hence, despite strong evidence that these proteins work together in a well-integrated molecular machine, mutations in individual components produce a surprisingly diverse collection of symptoms and diverse diagnoses.

The diversity of symptoms among cases and also differences in severity resembles the clinical picture observed for carriers of mutations in another class of functionally related proteins: the voltage-gated K^+, Na^+, and Ca^{2+} channels, together referred to as "channelopathies" (Kullmann, 2010). We propose to consider disorders emerging from mutations in the functionally related SNARE machinery "SNAREopathies"—a subset of the previously defined synaptopathies (Grant, 2012). However, the latter comprise a much more diverse group of genes; e.g., localized to different compartments (pre- and postsynaptic) and with diverse molecular functions. We define a SNAREopathy as a brain disease caused by mutations that disturb synaptic SNARE function; i.e., including disturbed functions of key regulators of SNARE function (Table 1; together referred to as "SNAREopathy genes"; Figure 1). Such a functional classification provides key advantages to (1) delineate a clinical subgroup with a common pathogenic starting point, (2) end the diagnostic odyssey for patient families, (3) contribute to elucidation of pathogenic pathways toward clinical neurodevelopmental phenotypes, and (4) eventually develop intervention strategies. The aim of this review

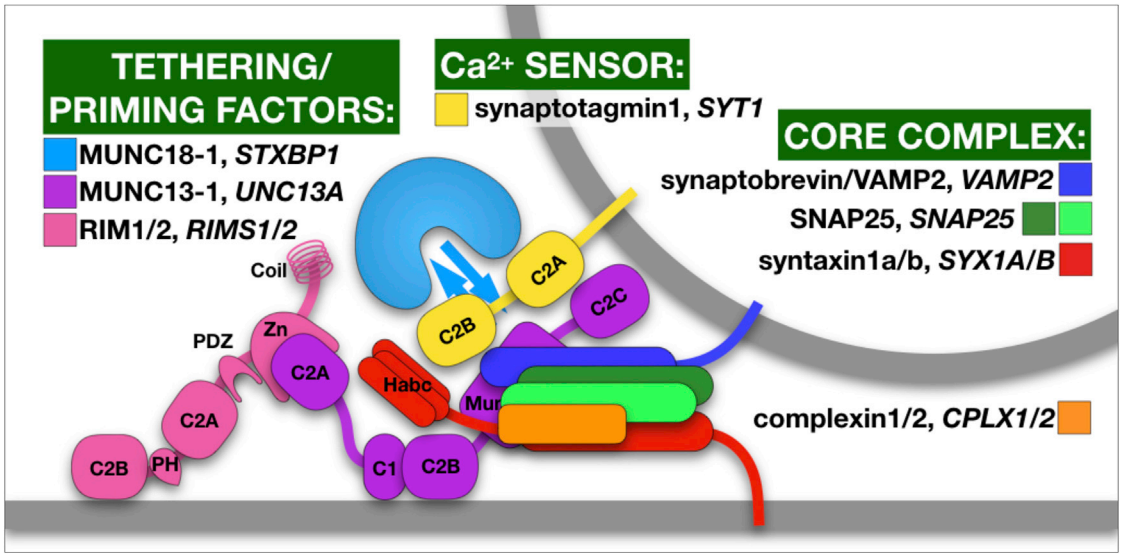

Figure 1. Schematic Representation of the Eight SNAREopathy Genes with Their Orientation Relative to the Synaptic Vesicle (Gray Circle) and the Plasma Membrane (Gray Line), Their Main Protein Domains, and Their Main Interactions

SNARE domains are depicted as aligned ellipses in the center. Indicated names are commonly used protein names followed by the official gene names separated by a comma. Firm evidence that mutations in a given gene specifically disturb synaptic SNARE function was taken as the main inclusion criterion for SNAREopathy genes. Hence, although only three of these eight SNAREopathy genes are SNAREs, all of them together serve a single purpose (SNARE-mediated membrane fusion), and mutations in any of these eight are expected to lead to a single convergent defect (dysregulation of SNARE-mediated membrane fusion). For additional genes (e.g., *CSP*, *CAPS1/2*, and *STXBP5/5L* [tomosyns]) and paralogs of the eight depicted genes (e.g., *VAMP1*, *SYT2*, and *UNC13B*), such evidence is already accumulating, and the collection of SNAREopathy genes is likely to expand in the future. Mutations in different paralogs may also affect neuronal or synaptic function in different populations of neurons; e.g., *UNC13A* for excitatory synapses (Augustin et al., 1999). Genes that regulate neuronal SNARE function as part of a generic role in multiple cellular trafficking routes or signal transduction cascades have been excluded; e.g., *αSNAP/NSF* and kinases and phosphatases.

is to define SNAREopathies, chart the landscape of resultant neurodevelopmental conditions with its complexity in symptoms and disease mechanisms, and suggest explanations for this diversity by considering partial redundancies and a heterogeneous effect among different neurons in the brain. Investigating these suggestions will improve connections between disturbed molecular function of the synaptic secretion machinery and the highly diverse clinical symptomatology and ultimately define outcome measures to evaluate future treatments.

Molecular Functions: SNAREopathy Genes Operate as a Highly Integrated Fusion Machine

SNARE complexes are dedicated membrane fusion machines, and different SNARE complexes participate in membrane fusion throughout the eukaryotic cell (Jahn and Scheller, 2006). In the presynapse, the neuronal SNARE complex is under control of additional proteins, some of which regulate SNARE complex assembly (MUNC18 and MUNC13), whereas others also regulate exocytosis downstream of SNARE complex formation (complexins and synaptotagmins). Functional studies have shown that formation of the SNARE complex drives vesicle "priming," the process that makes vesicles ready to be released, indicating that primed vesicles have already formed SNARE complexes (Walter et al., 2010). Downstream events triggered by Ca^{2+} and driven by synaptotagmins and complexins precipitates structural changes in the fusion machinery that trigger synaptic vesicle fusion and neurotransmitter release. Recent studies indicate that the initial step in formation of the SNARE complex consists of MUNC18-1 bound to syntaxin-1 in a closed configuration in which the SNARE motif formation is buried and unavailable for SNARE complex formation (Misura et al., 2000; Rizo and Südhof, 2012; Toonen and Verhage, 2007). Although, in this configuration, the SNARE motif is occluded, it forms the starting point for SNARE complex assembly (Ma et al., 2013; Schollmeier et al., 2011). MUNC13-1 disinhibits this autoinhibitory syntaxin: MUNC18-1 complex and enables interactions with VAMP2 (Parisotto et al., 2014; Sitarska et al., 2017). The subsequent SNARE complex formation takes place on the surface of MUNC18-1, which acts as a template. In addition, MUNC18-1 and MUNC13 prevent *trans*-SNARE complex disassembly, which would lead to "unpriming"—loss of primed vesicles (He et al., 2017). RIM proteins activate MUNC13 by preventing its autoinhibitory configuration and cluster Ca^{2+} channels with synaptic vesicles (Deng et al., 2011; Kaeser et al., 2011). Synaptotagmin-1 binds to the SNARE complex but might also participate in SNARE complex assembly, vesicle docking, and priming (Bhalla et al., 2006; Chang et al., 2018; de Wit et al., 2009; Schupp et al., 2016). Complexins bind to and stabilize the assembled SNARE complex and further stimulate fast synaptotagmin-driven membrane fusion (Cai et al., 2008; Reim et al., 2001). Synaptotagmins also inhibit spontaneous fusion (Courtney et al., 2019; Geppert et al., 1994), which is necessary to protect primed vesicles from premature fusion and build up a standing pool of primed vesicles (RRP, readily releasable pool of vesicles); a similar function is attributed to subdomains of complexins (Lai et al., 2014; Li et al., 2011; Makke et al., 2018; Maximov et al., 2009; Xue et al.,

Table 1. Clinical Features of SNAREopathies

SNAREopathy Genes	Null Mutant Phenotype in Mice	Null Mutant Phenotype Invertebrate	Number of Cases with Clinical Information	Seizures	EEG	Speech and language	Intellectual Disability	Motor/ Movement Disorders	Ophthalmic Disorders	Autism[a]/ Social Phenotypes	Other Symptoms	References
STX1B	die within 2 weeks (Wu et al., 2015); embryonic lethal if *STX1A* also abolished (Mishima et al., 2014; Vardar et al., 2016)	D Syx1A and C *Unc-64* mutants are lethal (Saifee et al., 1998; Schulze et al., 1995).	~50	febrile or afebrile seizures, variable severity	multifocal epileptic discharges, spike-wave discharges, etc.	often normal, affected in a few cases	none or mild to moderate	ataxia, dystonia, dysarthria, hypotonia, tremor	no	ASD in a few cases	–	Schubert et al., 2014; Vlaskamp et al., 2016; Wolking et al., 2019
STX1A	viable (Fujiwara et al., 2006; Gerber et al., 2008)	–	0–1	*STX1A* is often hemizygously deleted in Williams syndrome. STX1A expression correlates positively with intelligence within the syndrome. A homozygous splice-site mutation has been found in a patient with severe ID and muscular hypotonia. The consequence at the protein level is unknown.								Gao et al., 2010; Nakayama et al., 1998; Tassabehji et al., 1999; Reuter et al., 2017
SNAP25	lethal at birth (Washbourne et al., 2002)	D *SNAP25* null lethal at pharate stage (Vilinsky et al., 2002)	3	in all 3 patients	abnormal; epileptiform discharges etc.	profoundly affected	moderate to severe	hypotonia, myasthenia, ataxia	no (but ptosis)	not reported	hip dysplasia, scoliosis, joint deformities, apnea, ptosis	Hamdan et al., 2017; Rohena et al., 2013; Shen et al., 2014
VAMP2	lethal at birth (Schoch et al., 2001)	C *Snb-1* null lethal after hatching (Nonet et al., 1998), D *n-syb* null embryonic lethal (Deitcher et al., 1998)	5	in 3 of 5 patients	abnormal; e.g., sharp wave-slow wave or disorganized	profoundly affected	Moderate to severe	hypotonia, stereotyped hand movements, choreic movement in 3 of 5 patients	no	ASD	–	Salpietro et al., 2019
STXBP1	lethal at birth (Verhage et al., 2000)	D *Rop* null mutations lethal in larvae (Harrison et al., 1994; Wu et al., 1998), C *Unc18* null paralyzed (Sassa et al., 1999)	>250	95% of patients[b] early onset; spasms, focal or tonic seizures	epileptic activity, burst suppression; hypsarrhythmia	profoundly affected	severe to profound	hypotonia, ataxia, tremor, spasticity, dyskinesia, and dystonia are frequent	no	20% of published cases have autism or autistic features	extrapyramidal features in a few cases	Guiberson et al., 2018; Hamdan et al., 2011; Heyne et al., 2019; Lammertse et al., 2020; Lindy et al., 2018; Saitsu et al., 2008; Stamberger et al., 2016

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Table 1. *Continued*

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UNC13A	lethal within a few hours after birth (Augustin et al., 1999)	D *unc13* null lethal in embryo (Aravamudan et al., 1999), C *Unc13* nulls paralyzed (Richmond et al., 1999)	2	no	normal	delayed	mild	hyperkinesia, dyskinesia, repetitive behavior	no	ASD	ADHD	Engel et al., 2016; Lipstein et al., 2017
RIMS1	lethal if *RIMS2* also abolished (Schoch et al., 2006)	D and C *Rim* null animals viable, synaptic transmission reduced (Graf et al., 2012; Koushika et al., 2001)	~11	no	not known	normal	high IQ in mutated individuals of one family	not reported	cone-rod dystrophy	ASD	–	Dong et al., 2014; Iossifov et al., 2012; Johnson et al., 2003; Michaelides et al., 2005; Sisodiya et al., 2007
SYT1	lethal within a few hours after birth (Geppert et al., 1994)	D *Syt* null animals fail to hatch or are uncoordinated (Littleton et al., 1993)	11	no	epileptiform with high amplitude, low frequency	severely or profoundly affected	severe to profound	motor delay, infantile hypotonia, postinfantile dystonia, hyperkinesis	esotropia, strabismus, nystagmus, delayed visual maturation	limited interest in social interactions	switching between calm and excited states	Baker et al., 2015, 2018
Homozygous Mutations												
CPLX1	die within 4 months, lethal if *CPLX2* also abolished (Reim et al., 2001)	D. *cpx* nulls: most die before adult state (Huntwork and Littleton, 2007); C *cpx-1/cpx-2* double knockout: viable but uncoordinated (Huntwork and Littleton, 2007)	5	myoclonic epilepsy, often migrating, generalized seizures	epileptiform activity	profoundly affected	severe to profound	hypotonia, unable to crawl/walk or strongly delayed motor development	no	not reported	cortical atrophy reported in 2 siblings with the same mutation	Karaca et al., 2015; Redler et al., 2017

Shown is a summary of the main clinical features of SNAREopathies. ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; D, *Drosophila*; C, C. *elegans*.
[a]Probably underdiagnosed; diagnosis incidence may depend on how the patient families entered the healthcare system.
[b]More cases without seizures are now known, and some patients remain seizure free for years.

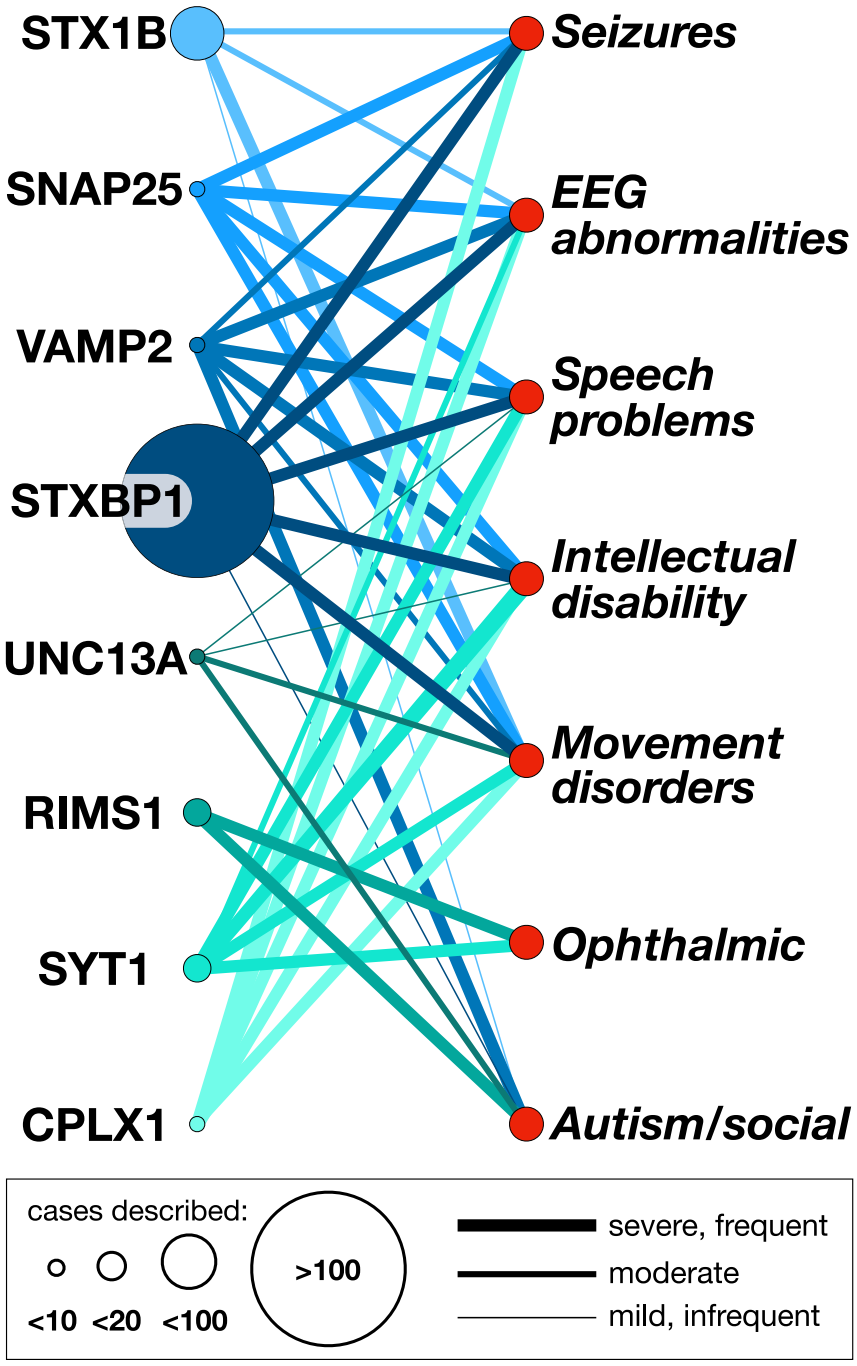

Figure 2. Prevalence of Symptoms for Mutations in Different SNAREopathy Genes

The thickness of the lines indicates the prevalence of reported symptoms and the size of the circles the number of reported cases to date. See Table 1 for details and references. The prevalence may be biased by how the patient families entered the health care system (e.g., likeliness that genetic testing is performed and that SNAREopathy genes are included in test panels).

2007), although this function is not observed in all cell types (Chang et al., 2015; Lopez-Murcia et al., 2019). Upon arrival of an action potential and Ca^{2+} binding, synaptotagmin acts as a switch to overcome the electrostatic energy barrier for fusion (Chang et al., 2018; Huson et al., 2019; Martens et al., 2007; Ruiter et al., 2019).

As part of the optimization of the fusion machine for speed, its components have become highly integrated, as demonstrated in elegant structural studies (Zhou et al., 2015, 2017). Hence, mutations in the genes encoding components of this highly integrated molecular machine are expected to lead to changes in the operation of the machine as a whole, and mutations in different components to lead to similar changes at the cellular level and, ultimately, to similar clinical manifestations.

Clinical Profiles: One Molecular Machine, Diverse Symptoms

In spite of this prediction, mutations in SNAREopathy genes lead to a strikingly diverse palette of early symptoms, often starting in the first year of life. A degree of pervasive NDD across language, cognitive, and motor milestones is the most common aspect, often accompanied by seizures, autistic features, spasms, ataxia, hypotonia, hyperkinesia, or stereotypies. For *RIMS1* and *SYT1*, characteristic ophthalmic abnormalities are reported (Table 1; Figure 2): for *RIMS1* mutations, primary photoreceptor defects (cone-rod dystrophy), whereas for *SYT1* mutations, eye positioning and movement are affected (strabismus and nystagmus).

In terms of prevalence, most cases are reported for MUNC18-1/*STXBP1*, more than 250; around 50 for *STX1B*; approximately 10 for *SYT1* and *RIMS1* (most are in the same family); a few for *SNAP25*, *VAMP2*, and complexin 1 (*CPLX1*); and two for *UNC13A* (Table 1; Figure 2). This prevalence is expected to increase rapidly because of increasing genetic testing worldwide, especially when these genes are added to the standard screening panels in neurodevelopmental clinical programs or when whole-exome sequencing is more generally introduced. Assembly of cohorts and systematic assessment of developmental trajectories and symptomatology has been complicated until now by the fact that patients enter the health care system via different routes in different countries (via pediatrics, clinical genetics, child neurology, or child psychiatry) and by the fact that the initial diagnoses are diverse. For instance, *STXBP1* mutations, first described in a patient diagnosed with Ohtahara syndrome (Saitsu

et al., 2008), were subsequently identified as causal in patients diagnosed with West syndrome, Dravet syndrome, Lennox-Gastaut syndrome, non-syndromic epilepsy, autism, and Rett-like syndrome (see https://stxbp1.cncr.nl/stxbp1_disorders and Stamberger et al., 2016, for further details). At present, treatment options are generally poor and limited to non-specific symptomatic treatments; e.g., seizure control. A more mechanistic understanding of the pathogenesis, especially when shared principles exist among mutations in different SNAREopathy genes, would be a major step toward rational therapy design.

Although the clinical picture for most cases is severe, in a few cases, symptoms are milder. For instance, mutations in *STX1B* often cause comparatively mild developmental delay, mild ID (intellectual disability), and febrile seizures. For *CPLX1*, all identified cases are homozygous children from consanguineous families carrying missense or nonsense mutations (Table 1). These parents have been described as unaffected, although more detailed phenotyping has not yet been reported (Redler et al., 2017). Together with the lack of ascertained patients with heterozygous mutations, *CPLX1* heterozygous mutations seem to be nonpathogenic.

Finally, a distinct class of mutations primarily affects the presynaptic part of the neuromuscular endplate, leading to congenital myasthenic syndrome (Lorenzoni et al., 2018), in some cases caused by a defect in the fusion machinery of acetylcholine vesicles. This machinery contains some of the same components as the prototypic CNS fusion machinery (Figure 1; Table 1) but, in some cases, paralogs; i.e., VAMP1 instead of VAMP2 and synaptotagmin-2 instead of synaptotagmin-1. Corresponding to this, mutations in *VAMP1* and *SYT2* result in ''peripheral SNAREopathy'' with hypotonia and areflexia and characteristic electrophysiological features, including a reduced compound muscular action potential that is further depressed during low-frequency stimulation but is facilitated upon high-frequency stimulation or voluntary contractions (Herrmann et al., 2014; Maselli et al., 2020; Salpietro et al., 2017; Whittaker et al., 2015).

Genetic Disease Mechanisms: Diverse Mechanisms Lead to Similar Syndromes

Most SNAREopathy cases are caused by heterozygous missense or LoF mutations, typically *de novo* but in rare cases inherited from heterozygous or mosaic parents (Saitsu et al., 2011). In addition to disease-causing mutations, recent large-scale sequencing projects (Lek et al., 2016) have revealed coding mutations in all SNAREopathy genes in healthy individuals (so-called population mutations). Population mutations may occur in parts of the protein where this is tolerated but also randomly throughout the gene and even in close proximity of disease-causing mutations (https://stxbp1.cncr.nl/stxbp1_disorders). In a few cases, mutations of the same amino acid are found in a patient as well as in a healthy individual (*STXBP1* V84). This suggests that other factors, genetic and/or environmental, should be considered when studying disease mechanisms.

The fact that mutations in genes that appear to have a single common function cause such a diverse palette of symptoms is a major challenge for understanding the underlying disease mechanisms. On top of that, these mechanisms appear to divert substantially among cases. Almost all SNAREopathies (except for *CPLX1*) described to date occur in patients who carry a normal allele and a pathogenic allele, which cannot be compensated for by the normal allele (haploinsufficiency). This situation can lead to dysfunction in several ways (Figure 3; Table 2): (1a) LoF or missense mutations reduce protein and/or mRNA stability, which brings the cellular level of the protein below a critical threshold without necessarily impairing its molecular function, or, conversely, (1b) missense mutations impair the function of a gene without necessarily making it less stable. These two situations might be functionally similar, but therapeutic strategies could be different (Figure 3; Table 2); e.g., stabilizing mutant proteins may be beneficial for mutations that make proteins less stable but do not affect functionality (1a), whereas such strategies will not work for those that do affect functionality (1b). As a further refinement, especially for larger, multi-domain proteins (1c), mutations may alter the interaction with one specific binding partner, yielding a protein with an abnormal interactome and, therefore, a different, new balance in functionality (neomorph). In such cases, future therapies might be specifically directed to normalize specific downstream pathways. In addition, (2) missense and LoF mutations can lead to dominant effects also affecting the functionality of the protein encoded by the other (normal) allele. In this case, therapeutic strategies might aim to downregulate or correct the mutant protein and/or transcripts. Finally, (3) many mutations are recessive and asymptomatic, but rare homozygous cases cause disease. Strikingly, evidence of all of these five scenarios has been reported for SNAREopathies (Figure 3; Table 2). Hence, not only is the palette of symptoms diverse, but the first indications of underlying disease mechanisms appear to be surprisingly diverse, too.

Disease-causing mutations in the three SNAREs VAMP2, STX1B, and SNAP25 are often point mutations clustering in the SNARE domains or in the essential H_{abc} domain of syntaxin 1b. This pattern indicates that disruption of the (canonical) SNARE function of these proteins probably explains the symptoms (scenario 1b or 1c), as demonstrated for a *VAMP2* missense mutation (S75P) in an *in vitro* fusion assay (Salpietro et al., 2019). Similarly, in synaptotagmin-1, five disease mutations cluster in the essential Ca^{2+}-binding C2B domain (Baker et al., 2018). One mutation (D304G) interferes with Ca^{2+} binding (scenario 1b or 1c), whereas another (M303K) affects the expression level (scenario 1a), and all mutations impair synaptic vesicle exocytosis and recycling (Baker et al., 2018). A thorough investigation of three synaptotagmin-1 disease mutations (D304G, D366E, and I368T) in mouse *SYT1*-null neurons showed that these mutant proteins fail to support synaptic transmission on their own, indicating profound LoF (Bradberry et al., 2020). When co-expressed with normal protein, to emulate the heterozygous condition found in patients, synaptic transmission was restored, but titration of the mutant/normal ratio revealed a dominant-negative phenotype (scenario 2). The cause of the defect was identified as impaired Ca^{2+}-dependent lipid binding, but to different extents in different mutants, which correlated with the severity of the clinical phenotype (Bradberry et al., 2020). In addition, one *SNAP25* mutation (I67N) has been shown to cause a reduction in vesicle fusion when overexpressed in wild-type mouse chromaffin cells, again indicating a dominant effect (scenario 2). This mutation has been shown to interfere with release

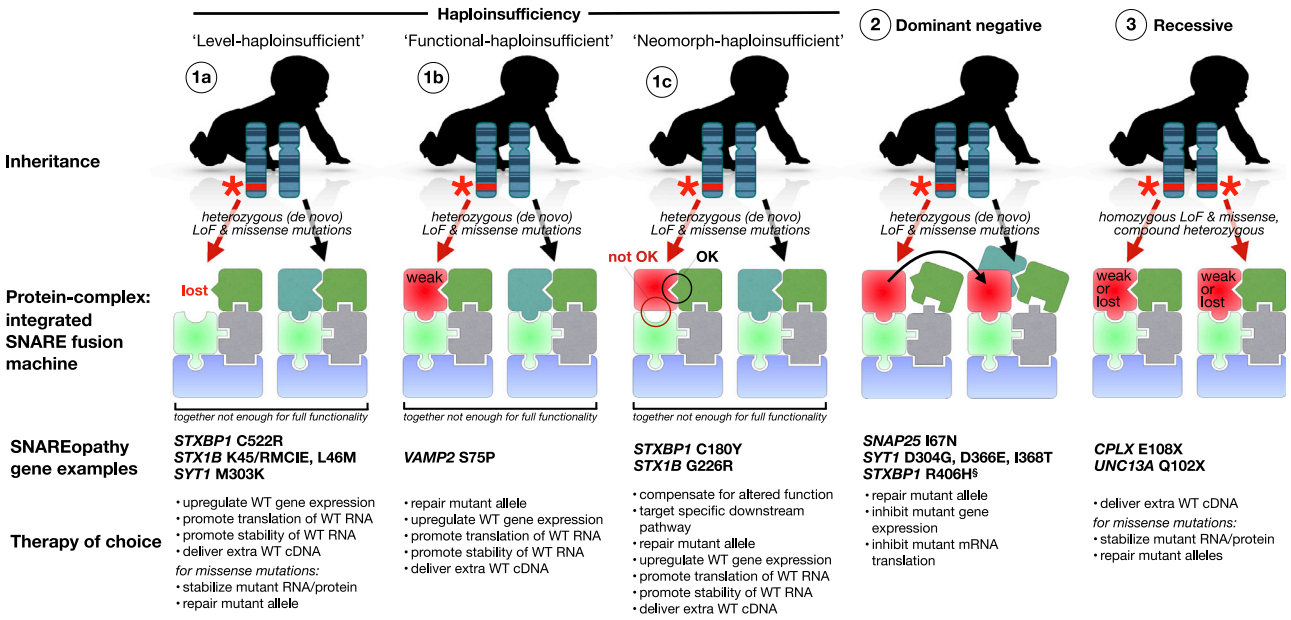

Figure 3. Five Molecular Genetic Disease Mechanisms for SNAREopathies

*LoF, classical LoF mutations; i.e., (partial) gene deletions and truncations (stop codons, frameshifts, or splice-site mutations); §, see discussion in the main text. The variation shown in this figure is restrained by lethality; i.e., more severe mutations, especially homozygous or compound heterozygous, are most likely not compatible with life. Furthermore, different types of haploinsufficiency (1a–1c) probably occur together; e.g., if mutations impair protein stability and functionality. Because all SNAREopathy genes have more than one established binding partner, detailed future studies will probably shift the proposed mechanisms from "functional haploinsufficient" (1b) to "neomorph" (1c) because mutations will probably affect interactions between binding partners differently, at least to some extent. One aspect not depicted in this scheme is the fact that SNARE complexes function in a cooperative manner, with several complexes involved in fusing a single synaptic vesicle with the plasma membrane. Dominant-negative effects may emerge when mutations interfere with this cooperativity, as demonstrated for an experimental mutation in *SNAP25* (Mohrmann et al., 2010). Two types of genetic mechanisms fall somewhat outside this classification. (1) Gain-of-function missense mutations are conceptually similar to functional or neomorph haploinsufficiency in heterozygous cases but, of course, cannot be considered a "weak" allele, as depicted here (example *UNC13A* P814L; Lipstein et al., 2017). Gain-of-function mutations can also be conceptually similar to recessive in homozygous cases; i.e., these mutations are only symptomatic in homozygous cases (example *STXBP1* L446F; Lammertse et al., 2020). (2) Proposed dominant-negative cases are those where a mutant protein self-aggregates, also incorporating the normal protein, as proposed for *STXBP1* (Guiberson et al., 2018); see discussion in the main text. Finally, to our knowledge, several possible genetic mechanisms have not yet been reported for SNAREopathies; e.g., increased gene copy number variants, variants that affect the gene promotor, mutations that affect the balance between splice variants, or cases where variation in more than a single SNAREopathy gene in a single individual contributes to the pathogenesis.

of energy from SNARE complex assembly (Rebane et al., 2018). Missense mutations (G226R and V216E) in the SNARE domain of syntaxin 1b have also been shown to change their interactome (scenario 1c), especially the binding to MUNC18-1 and MUNC13 (Vardar et al., 2020). The G226R mutation caused impaired binding to MUNC18-1 but an increased affinity for MUNC13 (scenario 1c) and also a reduced expression level (scenario 1a). When expressed in STX1A/B-deficient neurons, $STX1B^{G226R}$ produced a reduction in evoked post-synaptic current (EPSC) amplitude because of a smaller RRP (Vardar et al., 2020). The lower expression level did not seem to cause this phenotype, and therefore it corresponds to scenarios 1b and 1c (Figure 3; Table 2).

Strikingly, for *STXBP1* syndrome—the most frequent among SNAREopathies (Table 1)—about 60% of mutations described so far are truncations (nonsense, splice site, or frameshift) or partial or whole gene deletions (Stamberger et al., 2016) expected to reduce protein levels by 50%. This strongly suggests that insufficient expression is at the center of this disorder (scenario 1a). This even extends to missense mutations, which are found scattered throughout the gene without obvious clustering (Stamberger et al., 2016) and may impair protein stability and reduce protein levels by less than 50%. Seven missense mutations showed different cellular phenotypes when expressed in *STXBP1* null neurons, with five mutants rescuing neuron viability (Kovacevic et al., 2018) (which is compromised in *STXBP1*-null neurons; see Other Aspects of SNARE Function; Heeroma et al., 2004; Santos et al., 2017; Verhage et al., 2000). Expression levels at the synapse were consistently reduced, and further analysis of four mutants showed reduced protein stability. Indeed, reduced protein stability of missense mutations has been reported by several laboratories (Chai et al., 2016; Guiberson et al., 2018; Kovacevic et al., 2018; Martin et al., 2014; Saitsu et al., 2010). It has also been reported that some missense mutants, in addition to reduced stability, also form protein clusters that might trap wild-type protein (Chai et al., 2016), resulting in its degradation (Guiberson et al., 2018). This would imply a dominant-negative scenario (scenario 2) (Guiberson et al., 2018). Accordingly, expressing mutant protein on a wild-type background in *C. elegans* resulted in mild impairment of neurotransmitter release (Guiberson et al., 2018). However, overexpression of missense mutants in murine *STXBP1* heterozygous neurons did not affect neurotransmission (Kovacevic et al., 2018), and humans heterozygous for partial or complete *STXBP1* deletions or

Table 2. Genetic Disease Mechanisms

Modes		Main Mutation Types	Therapeutic Strategies	SNAREopathy Examples	References
(1) Haploinsufficient a	level haploinsufficiency: mutations reduce protein and/or mRNA level and stability; cellular protein level below critical threshold. Function of mutant protein may not be altered.	heterozygous loss-of-function (LoF) mutations (deletions, truncations, frameshifts, splice-site mutations) and missense mutations, no hotspots	upregulate the unaffected allele, promote translation of unaffected RNA, deliver exogenous cDNA; missense mutations: stabilize mutant RNA and protein	*STXBP1 C522R* *STX1B K45/RMCIE, L46M* *SYT1 M303K*	Kovacevic et al., 2018; Vardar et al., 2020; Baker et al., 2018
(1) Haploinsufficient b	functional haploinsufficiency: mutations change (typically impair) protein function (without necessarily making mRNA or protein less stable)	heterozygous missense mutations, often clustered in the main functional domain	gene repair, compensate for altered function, deliver exogenous cDNA	*VAMP2 S75P* *UNC13A P814L (gain of function)*	Lipstein et al., 2017; Salpietro et al., 2019
(1) Haploinsufficient c	neomorph haploinsufficiency: mutations alter interaction with specific binding partner, yielding abnormal interactome, functionality balance	heterozygous missense mutations in one of several functional domains	target specific downstream pathway, deliver exogenous cDNA	*STXBP1 C180Y* *STX1B G226R*	Saitsu et al., 2008; Vardar et al., 2020; Baker et al., 2018
(2) Dominant-Negative	mutations affect the function of the wild-type protein by interfering with cellular interactions, functions	heterozygous LoF and missense mutations	inhibit mutant gene, RNA, protein expression	*SNAP25 I67N* *STXBP1 R406H*[§] *SYT1 D304G, D366E, I368T*	Rebane et al., 2018; Guiberson et al., 2018
(3) Recessive	mutations cause no (strong) symptoms in heterozygous carriers	homozygous LoF and missense mutations or compound heterozygous LoF/missense mutations	gene repair, deliver exogenous cDNA	*STXBP1 L446F* *CPLX E108X* *UNC13A Q102**	Lammertse et al., 2020; Karaca et al., 2015; Engel et al., 2016

Shown are different molecular genetic disease mechanisms relevant for SNAREopathies; see also Figure 3. Combinations of these situations are also plausible (and have been reported; e.g., 1a + 1b/c). Increased gene copy number has not been reported, nor increased stability. See discussion in the main text.

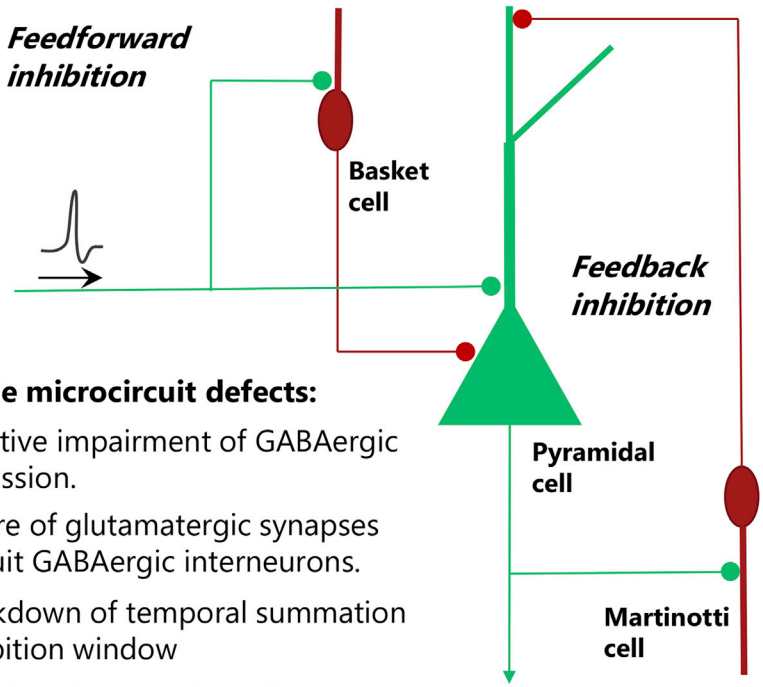

Figure 4. Possible Microcircuit Defect in SNAREopathies

We hypothesize that microcircuit failure, especially at high stimulation frequencies, might explain pervasive clinical symptoms, such as ID and seizures. Feedforward inhibition takes place via an intercalated parvalbumin-positive basket cell, whereas feedback inhibition occurs via activation of a somatostatin-positive Martinotti cell. The defects in the microcircuit might either affect GABAergic neurotransmission (1), glutamatergic neurotransmission (2), or breakdown of temporal summation or inhibition windows (3). Finally, selective degeneration of a neuronal subtype (4) might underlie disease, although this remains unknown.

truncations show similar clinical phenotypes as heterozygotes of missense mutations (Stamberger et al., 2016), whereas a dominant mechanism commands that missense mutations are more deleterious. Hence, the present data indicate a striking and so far unexplained complexity where mutations in *STXBP1* typically lead to too low of an expression level (scenario 1a), whereas mutations in the other proteins lead to impaired functionality (scenario 1b/1c or 2), and only occasionally are expression levels affected, as demonstrated for one *SYT1* mutation (Baker et al., 2018) and an insertion or deletion mutation in *STX1B* (Vardar et al., 2020).

Occasionally a missense mutation leads to a protein with gain-of-function characteristics; i.e., a gain-of-function neomorph (scenario 1c). A *UNC13A* case (P814L) carries a gain-of-function mutation (increased fusion probability), as indicated by experiments in mouse neurons (Lipstein et al., 2017), but still causes a motor disorder, mild ID, and autism. A gain-of-function homozygous missense *STXBP1* mutation (L446F) has been reported recently in two siblings diagnosed with ID, epilepsy, and electroencephalogram (EEG) abnormalities (Lammertse et al., 2020). Upon expression in *STXBP1*-null mouse neurons, evoked synaptic transmission and the vesicular release probability were increased 2-fold. The disease-causing V216E mutation in *STX1B* is located next to a "furled loop" in MUNC18-1, which regulates the autoinhibitory configuration of the STX1B-MUNC18-1 complex (Vardar et al., 2020). The mutation causes increased EPSC size and vesicular release probability. In all three gain-of-function mutations, the RRP size was unchanged, and the increased release probability was associated with changes in short-term plasticity (increased depression). In spite of these striking cell-physiological similarities, the clinical features of the patients were diverse. The *STXBP1* L446F patients had severe epilepsy (diagnosed as Lennox-Gastaut syndrome; Lammertse et al., 2020)), the *UNC13A* patient was autistic and without known epilepsy (Lipstein et al., 2017), and the *STX1B* V216E patient had cognitive impairment, macrocephaly, and ataxia (Vardar et al., 2020). This underscores the marked difference in clinical features in spite of mutations with seemingly similar cell-physiological consequences.

Explaining Complexity I: Symptom Severity Generally Scales with Genetic Redundancy

The remarkable complexity in symptoms and genetic mechanisms among SNAREopathy cases suggests that additional genetic and/or environmental factors contribute substantially to disease expression. Therefore, it seems crucial to consider the rest of the genome when studying individual cases and, e.g., study patient-own models. In addition, to start understanding this striking complexity, at least two scenarios may be considered: (1) functional redundancy may differ between SNAREopathy cases, and (2) subtle differences may exist in how different components of the SNARE fusion machine are rate-limiting in different types of neurons.

Genetic redundancy, the principle that other genes compensate partially or wholly for functionality loss of another gene, has been extensively studied for all SNAREopathy genes in null mutant (knockout [KO]) mice, which typically display perinatal lethality: *SNAP25* (Washbourne et al., 2002), *STXBP1*/MUNC18-1 (Verhage et al., 2000), *VAMP2* (Schoch et al., 2001), *UNC13A* (Augustin et al., 1999), *STX1A* (Wu et al., 2015), *STX1A/B* double KO (Vardar et al., 2016), *RIMS1/2* double KO (Schoch et al., 2006), and *CPLX1/2* double KO (Reim et al., 2001) (Table 1). Lethality upon disruption of SNAREopathy genes extends to invertebrates (Table 1 and references therein). This indicates that the protein machinery of evoked neurotransmitter release displays very limited redundancy. Although paralogs exist, they typically do not perform in what matters most at the synapse: fast

("synchronized") release of neurotransmitters. For instance, expression of exogenous SNAP23, the closest relative of SNAP25, substitutes for SNAP25 in several functions, including neuronal survival and fusion of DCVs, and even priming and spontaneous and slow ("asynchronous") fusion of synaptic vesicles, but SNAP23 does not support fast synchronized release (Arora et al., 2017; Delgado-Martínez et al., 2007). Similarly, upon elimination of synaptotagmin-1 or -2 expression, which are dedicated to fast synchronized release, the co-existence of synaptotagmin-7 allows slow ("asynchronous") release to persist (Bacaj et al., 2013; Schonn et al., 2008) but at a level incompatible with life (Geppert et al., 1994; Pang et al., 2006). Yet another example is that exogenous MUNC18-2 (or even the more distantly related MUNC18-3) substitute for several cellular functions of MUNC18-1 KO neurons, including cell viability, but, again, fast synchronized release is almost eliminated because of destabilization of the primed vesicle state (Gulyás-Kovács et al., 2007; He et al., 2017). Thus, although the neuronal SNARE complex and its associated proteins perform a function (membrane fusion) that is similar in other membrane trafficking processes in the cell, the neuronal fusion apparatus is so specialized and integrated that none of the paralogs can really substitute. Thus, the major effect of single mutations in several SNAREopathy genes is the price paid for extensive specialization and optimization of fast synaptic transmission.

However, in the case of syntaxin-1, the paralogs STX1A and STX1B have similar functionality in synaptic transmission, and mutations in *STX1B* often cause comparatively mild symptoms: febrile seizures and mild NDD and disability, which might be explained by partial redundancy with *STX1A*. Consistent with this, currently no *STX1A* mutations are described in relation to encephalopathies (one reported case awaits causal evidence; Table 1). The redundancy is evident from the fact that *STX1A/B* double KO mice die in the embryonal state (Mishima et al., 2014; Vardar et al., 2016), whereas *STX1B* KO mice die within 2 weeks (Mishima et al., 2014; Wu et al., 2015), and *STX1A* KO mice are viable (Fujiwara et al., 2006; Gerber et al., 2008; Table 1). Consistent with the idea that redundancy predicts severity, invertebrates that express only one syntaxin-1 ortholog display early lethality upon disruption of the gene (Saifee et al., 1998; Schulze et al., 1995; Table 1). In the case of complexins, two paralogs support fast synaptic transmission: *CPLX1* and *CPLX2*. *CPLX1* deficiency in mice leads to death within 4 months (Reim et al., 2001), whereas *CPLX2* KO mice are viable (Reim et al., 2001), but double-null mutants die immediately after birth (Reim et al., 2001), again indicating redundancy. For *CPLX1*, all patients identified so far are homozygous children from consanguineous families carrying missense or nonsense mutations (Table 1), and the parents are described as asymptomatic (Redler et al., 2017). Together with the lack of any known patients with heterozygous mutations, this indicates that, for *CPLX1*, heterozygous mutations are tolerated, most likely because of partial redundancy with *CPLX2*. Similarly, for *UNC13A* and *RIMS1*, genes for which close paralogs exist that (partially) compensate for the loss of the proteins in mice (see above), the symptoms in human carriers with mutations in these genes are relatively mild (Table 1). Hence, redundancy helps to explain the fact that *STX1B*, *UNC13A*, and *RIMS1* cases are relatively mild and *CPLX* cases are homozygous. Generally, disease severity scales with how much redundancy is observed; e.g., in genetic studies of mice.

Explaining Complexity II: Heterogeneous Effect in Neuronal Networks

Although the overall clinical severity appears to correlate with functional redundancy (versus the lack thereof), how SNAREopathy mutations lead to neurodevelopmental impairments remains unexplained. Subtle differences in rate-limiting steps in synaptic transmission in different neuronal subtypes, together with the organization of brain micro-circuits, provide a starting point to explain this. Among the main developmental symptoms, seizures, intellectual disability, and autism spectrum disorder (ASD) are common and have all been linked to dysregulation of the excitation/inhibition (E/I) balance in brain circuits (Golden et al., 2018). At first glance, the SNARE machinery appears to be universally required in all synapses, glutamatergic (excitation) and GABAergic (inhibition). Thus, the etiology of the disease does not predict E/I dysregulation. However, glutamatergic and GABAergic neurotransmission may display slightly different susceptibilities to SNAREopathy gene mutations. For instance, inhibitory synaptic transmission showed stronger rundown than excitatory transmission during high-frequency train stimulation in *STXBP1*$^{+/-}$ (haploinsufficient) neurons *in vitro* (Toonen et al., 2006). Such a differential effect is expected to cause hyperexcitation during high-frequency bursting. In a recent study of *STXBP1* haploinsufficient mice, impaired inhibition was identified in cortical brain slices (Chen et al., 2020). The mechanisms depended on the interneuron type. In parvalbumin-positive (basket) neurons (PV neurons), connectivity rates to pyramidal neurons were normal, but the unitary connection strength was reduced, whereas for somatostatin-positive (Martinotti) neurons, the connectivity rates were reduced, but the unitary strength was normal; for both interneuron types, short-term plasticity was unaffected (Chen et al., 2020). Conversely, another study of *STXBP1*$^{+/-}$ mice concluded that seizures in these mice are triggered by a reduction in cortical excitatory neurotransmission to fast-spiking interneurons in the striatum (Miyamoto et al., 2019). In this case, *STXBP1* haploinsufficiency would essentially be a disease of cortical glutamatergic synapses, resulting in failure to recruit GABAergic interneurons. Given these observations in mouse model neurons, it is plausible that altered (impaired) SNAREopathy gene function affects different components in neuronal networks in the brain to a different extent, creating an imbalance. Such a scenario has also been proposed for channelopathies where causative genes are also expressed in excitatory and inhibitory neurons, whereas mutations do cause syndromic epilepsies (Staley, 2015).

A more specific explanation of SNAREopathy symptoms requires a deeper understanding of the extent to which the different components of mixed glutamatergic-GABAergic microcircuits in the brain are susceptible to SNAREopathy gene mutations; e.g., feedforward and feedback inhibition circuits (Figure 4). Feedforward circuits are found in the cortex, hippocampus, and striatum, among others. Because inhibition involves two synapses in series (di-synaptic), whereas excitation is monosynaptic, excitation of the circuit results in a brief time window within which the principal cell can sum up multiple excitatory inputs and elicit an action potential (Hu et al., 2014; Pouille and Scanziani, 2001). A generalized synaptic impairment might affect this circuit in multiple ways. It might disproportionally

affect PV interneurons, resulting in failure of inhibition (Figure 4, possibility 1; Chen et al., 2020), or impaired excitatory transmission could impair recruitment of the PV interneuron (Figure 4, possibility 2; Miyamoto et al., 2019). Breakdown of inhibition should be considered not only in terms of its magnitude but also in terms of its timing (Figure 4, possibility 3); a break-down in synchronicity or inconsistent activation of interneurons could potentially lead to prolonged or ill-defined summation windows. We hypothesize that an impaired summation window might be correlated with ID, which is a particularly consistent feature of SNAREopathies (Figure 2; Table 1). For instance, all *STXBP1* mutation carriers are diagnosed with ID, whereas epilepsy is not found in all patients (Stamberger et al., 2016). This kind of impairment might fundamentally compromise the brain's processing ability, leading to ID. It could also cause defects in voluntary movement, another computational-intensive activity, which is consistently affected (Table 1). Initially, homeostatic mechanisms might compensate for the impaired neurotransmitter release anticipated for most mutations and normalize synaptic transmission (Fernandes and Carvalho, 2016; Turrigiano, 2012; Vitureira et al., 2012). However, at higher frequencies, presynaptic impairments will lead to microcircuit failure. Similar impairments in synaptic homeostasis could be envisioned in feedback inhibition circuits, which, in the cortex, typically involve somatostatin-positive Martinotti cells (Silberberg and Markram, 2007; Figure 4).

To better understand how SNAREopathy mutations affect the stability of brain networks, it seems crucial to trace effects back to their origin and establish how presynaptic impairments affect early brain development (e.g., microcircuit and system connectivity) and the timing of known maturation checkpoints, such as the GABA/Cl^- switch and subunit switches in GABA and glutamate receptors. Although SNAREopathy genes are not known to have a prominent role in early development (see below), the earliest, subtle effects certainly affect all subsequent stages of brain development but happen long before carriers enter the healthcare system, and it seems impossible to backtrack to the initial dysregulation in the carriers themselves. Mouse models with good construct and face validity may help to obtain leads toward such early events.

Other Aspects of SNARE Function

Many studies emphasize the central role of the eight SNAREopathy genes in synaptic vesicle fusion and synaptic transmission, but other biological functions have also been demonstrated for these genes. Dysregulation of these functions because of pathogenic mutations may also contribute to SNAREopathy pathogenesis and potentially also to the diversity in clinical manifestations because the contribution of these eight genes to other processes might be more diverse than for synaptic transmission.

First, SNAREopathy genes have been proposed to have a role in axon outgrowth during brain development (Verhage et al., 2000, and references therein; Hamada et al., 2017; Yamamoto et al., 2019). Human mutation carriers show early symptoms, consistent with dysregulation during initial brain development. However, *in vivo* studies in null mutant mice for SNAREopathy genes (see references in Table 1) indicate that the brain develops normally, including correct targeting of the main nerve bundles and normal connectivity, in the absence of SNAREopathy proteins. This suggests that SNAREopathy genes are not major factors in establishing the initial neuronal networks in the brain during early brain development.

Second, SNAREopathy genes are also crucial for regulated secretion of neuropeptides and neuromodulators from dense core vesicles (Arora et al., 2017; Farina et al., 2015; Persoon et al., 2019; Shimojo et al., 2015; van de Bospoort et al., 2012; van Keimpema et al., 2017). Neuromodulators are important factors in the adult brain but also during maturation of brain networks, consistent with the early symptoms observed in human mutation carriers. Neuromodulator and neuropeptide secretion is a distinct secretory pathway for which much less information is available than for synaptic vesicle exocytosis. The first indications suggest that SNAREopathy genes operate in a similar concerted manner as for synaptic vesicle exocytosis and that dysregulation of this pathway may well contribute to SNAREopathies but may not provide direct explanations for symptom diversity.

Finally, three SNAREopathy genes, *STXBP1*/MUNC18-1, and *SNAP25*, *STX1A/B*, but not the other five, are known to be essential for neuronal survival (Santos et al., 2017). Mutant mice or primary neurons in culture show massive neuronal cell death in the absence of the encoded proteins (Arora et al., 2017; Santos et al., 2017; Vardar et al., 2016; Verhage et al., 2000). This essential role in neuronal survival and/or maintenance is distinct from their established role in synaptic transmission because (1) expression of non-neuronal paralogs prevents cell death but does not restore synaptic transmission (Santos et al., 2017) and (2) other mutant mice and/or neurons that lack synaptic transmission do not show cell death (Schoch et al., 2001; Varoqueaux et al., 2002). Sparse inactivation of the *STXBP1* gene in mice shows that its role in neuronal survival/maintenance is cell autonomous (Heeroma et al., 2004). Hence, in addition to the predicted effect of SNAREopathy gene mutations on synaptic transmission, effects on neuronal survival and/or maintenance cannot be excluded, and SNAREopathies might, in some cases, involve progressive loss of neuronal subpopulations (Figure 4, possibility 4). However, so far, studies performed *in vitro* failed to demonstrate a difference in neuronal survival for *STXBP1* (Kovacevic et al., 2018; Patzke et al., 2015; Verhage et al., 2000) or *STX1B* heterozygous neurons (Vardar et al., 2016), indicating that neuronal survival is more robust to mutations than synaptic transmission.

Conclusions and Perspective

Advances in clinical genetics have led to widespread genomic testing of children with severe neurodevelopmental disorders and an explosion of new clinicopathological connections. Patients with only partially overlapping clinical features and diverse diagnoses have been found to share mutations in the core components of the synaptic SNARE fusion machinery. Collectively defining these cases, based on etiology and mechanism, as SNAREopathies provides key advantages for a more systematic analysis as a clinical subgroup with a common pathogenic starting point to end the diagnostic odyssey for patient families, promote elucidation of common pathogenic pathways, and develop intervention strategies. Mutations in SNAREopathy genes cause

a strikingly diverse palette of symptoms, and the first mechanistic studies demonstrate that the genetic and cell-physiological disease mechanisms are also surprisingly diverse. Clinicians and basic scientists must work together to find commonalities among cases, at different stages of brain development and different organizational levels in the brain (synapses, neurons, networks, and brain systems), to understand how different clinical manifestations emerge from a common pathogenic starting point (mutations in a single, integrated molecular machine) and to design rational intervention strategies. We argue that disease severity generally scales with how much functional redundancy is available for individual SNARE components and that subtle differences in susceptibility to SNAREopathy mutations in different components of neuronal networks provide a starting point for understanding neurodevelopmental manifestations. To achieve a more complete understanding, we need to (1) systematically analyze the links between the molecular disease mechanism (as revealed in standardized *in vitro* systems) and dysregulation at the level of the intact circuitry of the brain and (2) generate relevant and diverse *in vitro* and *in vivo* disease models that better recapitulate the diversity seen among affected humans. Given the diversity in symptoms even among carriers of mutations in a single SNAREopathy gene, it seems crucial to study patient-own model systems (e.g., induced pluripotent stem cell [iPSC]-derived human neurons; Fenske et al., 2019; Meijer et al., 2019; Rhee et al., 2019) and (3) follow *in vivo* disease models over longer time spans to assess the developmental and possible neurodegenerative aspects of the diseases, which account for a part of patient variability. Finally, (4) clinical analyses should focus on further expanding the detailed characterization of developmental trajectories and symptomatology and explore rational and symptomatic treatment improvement. Finally, the implication of network-level E/I deregulation as a downstream consequence of SNAREopathies may be tested by clinical measures of brain function, such as EEG. In any scenario, a clinical developmental natural history study is indispensable to evaluate the success of future therapies.

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DECLARATION OF INTERESTS

M.V. participates in a holding that owns shares of Sylics (Synaptologics BV), a private company that offers STXBP1 and SNAP25 disease modeling, and has received consulting fees from Sylics.

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